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(54) Title: METHODS FOR PRODUCING GONADOTROPIN AND TSH SUPER-AGONISTS

(57) Abstract

Methods are provided for producing super-agonist hormones, which hormones are those having the common alpha subunit of the gonadotropins, generally comprising the transformation of host cells with recombinant DNA comprising a first DNA sequence coding for an alpha subunit from an equine animal species and a second DNA sequence coding for a beta subunit from a non-equine species. The preffered hormone comprises an equine alpha subunit and a bovine, porcine or human beta subunit of LH, FSH, CG or TSH and exhibits a greatly increased activity.

EQUINE ALPHA SUBUNIT cDNA AND AMINO ACID SEQUENCE

```
   1 GGGGGGGGGGGGGGGGGGGGCTGCTCTGAACACATCCTACAAAAAGTCCAGAGGAAGAAG   60

                MetAspTyrTyrArgLysHisAlaAlaValIleLeuAlaThrLeuSerValPheLe
  61 AGCCATGGATTACTACAGAAAACATGCAGCTGTCATCCTGGCCACATTGTCCGTGTTTCT  120

                uHisIleLeuHisSerPheProAspGlyGluPheThrThrGlnAspCysProGluCysLy
 121 GCATATTCTCCATTCCTTTCCTGATGGAGAGTTTACAACGCAGGATTGCCCAGAATGCAA  180

                sLeuArgGluAsnLysTyrPhePheLysLeuGlyValProIleTyrGlnCysLysGlyCy
 181 GCTAAGGGAAAACAAGTACTTCTTCAAACTGGGCGTCCCGATTTACCAGTGTAAGGGCTG  240

                sCysPheSerArgAlaTyrProThrProAlaArgSerArgLysThrMetLeuValProLy
 241 CTGCTTCTCCAGAGCGTACCCCACTCCAGCAAGGTCCAGGAAGACAATGTTGGTCCCAAA  300

                sAsnIleThrSerGluSerThrCysCysValAlaLysAlaPheIleArgValThrValMe
 301 GAACATCACCTCAGAATCCACATGCTGTGTGGCCAAAGCATTTATCAGGGTCACAGTCAT  360

                tGlyAsnIleLysLeuGluAsnHisThrGlnCysTyrCysSerThrCysTyrHisHisLy
 361 GGGAAACATCAAGTTGGGAGAACCACACCCAGTGCTATTGCAGCACTTGCTATCACCACAA  420

                sIleEnd
 421 GATTTAAATGTTTCACCAAGTGCCTTGTGGATGACTGCTGATTTCCACCCCCCCCCCCCCC  480
```

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METHODS FOR PRODUCING GONADOTROPIN AND TSH SUPER-AGONISTS

Field of Invention

This invention relates to the expression, by recombinant DNA technology, of unique gonadotropin hybrid molecules. Gonadotropin molecules are dimeric in nature, containing a common alpha subunit and a hormone-specific beta subunit. This application describes the technology to produce large amounts of a gonadotropin that is composed of an alpha subunit ordinarily found in one animal and the beta subunit from another species.

Background of Invention

It has been known for almost 20 years that the glycoprotein hormones luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) are dimeric molecules consisting of a common alpha subunit and a hormone specific beta subunit (G. Bousfield et al., Mol. Cell. Endoc. 40:67-77 [1985]; T-H Liao et al., J. Biol. Chem. 245:3275-3281 [1970]; J.G. Pierce et al., Ann. Rev. Biochem. 50:465-495 [1981]). While the genes that encode each of the three beta subunits are unique and have different chromosomal assignments, a single gene is responsible for the production of the same alpha subunit for each hormone (J.C. Fiddes et al., In: Bioregulators of Reproduction, Acad. Press. pp279-304 [1981]). Recombination of the beta subunit of LH, FSH or TSH with the alpha subunit of another

results in the production of a hybrid molecule with biologic activity of the beta subunit (G. Bousfield et al., Mol. Cell. Endoc. 40:67-77 [1985]; L.E. Reichert et al., Endocrin. 87:531-534 [1970]; J.G. Pierce et al., J. Biol. Chem. 246:2312-2324 [1971]). Thus, dissociation of bovine LH into the alpha and beta subunits and mixing of the bovine LH alpha with free bovine beta TSH will produce a hybrid molecule with bovine TSH bioactivity.

It was also noted that free subunits obtained from dissociated hormones exhibit little or no biologic action when added to target cells (K.J. Catt et al., J. Clin. Endocrin. Metabol. 36:73-79 [1973]; J.F. Williams et al., Endocrin. 206:1353-1359 (1980]). Only dimers of the alpha and beta subunits are biologically active. Studies have demonstrated that both the alpha and beta subunits must interact with the receptor to elicit a biological response (W.R. Moyle et al., Proc. Natl. Acad. Sci. USA 79:2245-2250 [1982]). Further, it has been demonstrated that the carbohydrate moieties present upon the subunits (in particular, the alpha subunit) are essential for transduction of this extracellular hormonal signal to an intracellular biological action, usually through the action of the adenylate cyclase system (M.R. Sairam et al., Science 229;65-67 [1985]).

Scientists have examined the effects of recombination of subunits obtained from hormones of different animals. When the amino acid sequence is compared between the ovine, bovine, porcine, and human alpha subunits, a striking sequence homology is observed (J.G. Pierce et al., Ann. Rev. Biochem. 50:465-495 [1981]). It has been demonstrated that human alpha subunit and

ovine LH beta subunit, when combined, exhibited LH bioactivity (L.E. Reichert et al., Endocrin. 87:531-534 [1970]). As a result of the gene conservation of gonadotropin subunits between species, almost all inter-species hybrid molecules have been shown to be biologically active. Many other studies have confirmed that recombining subunits of LH obtained from different species resulted in the production of hormones with approximately equivalent biologic activities (T-H Liao et al., J. Biol. Chem. 245:3275-3281 [1970]; L.E. Reichert et al., Endocrin. 87:531-534 [1970]; J.G. Pierce et al., Ann. Rev. Biochem. 50:465-495 [1981]).

Much less is known about structure-function relationships of equine gonadotropins. Equine LH and FSH have recently been purified from horse pituitary glands and the biochemical structure and biologic activity of the molecules studied. These molecules exhibit some unique features. First, equine chorionic gonadotropin (the hormone of pregnancy analogous to human chorionic gonadotropin) exhibits both LH and FSH bioactivity (Y. Combarnous et al., Endocrin. 115:1821-1827 [1984]). Secondly, equine FSH exhibits much greater potency in a number of bioassay systems, when compared on a molar basis, to rat or porcine FSH (F. Guillou et al., Biochem. Biophys. Acta. 887:196-203 (1986])). Thirdly, the equine LH molecule exhibits at least 100 times greater bioactivity on a molar basis than LH molecules purified from sheep or porcine pituitaries (G. Bousfield et al., Mol. Cell. Endocrin. 40:69-77 [1985]). Finally, the equine LH beta subunit exhibits a C-terminal extension that is rich in serine-linked (O-linked) oligosaccharides, unlike other LH subunits, but quite

similar to the C-terminal peptide of hCG (G.R. Bousfield et al., J. Biol. Chem. 259:1911-1921 [1984]).

When hybrid gonadotropin molecules were formed with purified equine subunits combined with the corresponding subunits of other species, yet another interesting phenomenon was observed. A hybrid LH molecule, that contains the alpha subunit of equine and the bovine or porcine β subunit of LH, results in an LH molecule with 10-40 times the biopotency of the corresponding homologous LH molecule. No teaching, however, was provided in order to produce such molecules in useful quantities or purities.

In the original paper that describes the unusually amplified bioactivity of hybrid LH molecules with equine alpha (up to 49 times as active as the homologous LH species) the authors suggest that this phenomenon may be the result of the unique tyrosine-histidine transposition observed at the C-terminus of this equine subunit (G. Bousfield et al., Mol. Cell. Endocrin. 40:69-77 [1985]).

Superovulation of cows and other animals is performed routinely to expand herd size with genetically superior animals. Induction of superovulation with a hormone of greater bioactivity would be highly desired since such require less hormone, thus reducing costs. While it appears that a hybrid gonadotropin might accomplish this, only conventional biochemical methods are available to produce these unique hybrid molecules. Such methods would rely upon pituitary glands obtained from horses and cows which

could not be supplied in adequate quantities for continuous commercialization. Additionally, the cost of purification, dissociation, rehybridization and purification would be much too great to be of conventional value.

It is one aspect of the present invention to provide methods and technology to produce large amounts of a superactive hybrid agonist of bovine FSH.

As the activity of the beta subunit can be greatly enhanced by the addition of an equine alpha subunit, it should be understood that any known utility of proteins having the common alpha subunit of the gonadotropins, such as human chorionic gonadotropin, luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone, in any species other than equine, can be greatly enhanced by creating a hybrid molecule with an equine alpha subunit and the beta subunit of the hormone of interest. Thus, the treatment of sterility in humans can also be greatly enhanced by using a chimeric hybrid of human FSH, for example, and an equine alpha unit.

Thus, it is another aspect of the present invention to provide methods and technology to produce large amounts of a super-active hybrid agonist of any hormone having the common alpha subunit of the gonadotropins, including human hormones.

Summary of Invention

In accordance with these aspects of the instant invention, there are provided new methods for producing large quantities of super-active hormones having the common alpha subunit of

the gonadotropins, such as LH, FSH, CG, and TSH, and preferably bovine gonadotropins, utilizing recombinant DNA methods. The preferred molecules advantageously would be used in the superovulation of bovine animals for the breeding of preferred traits. The new methods of the instant invention produce the hybrid molecule (preferably equine α/bovine LH β or, more preferably, bovine FSH β) super-agonist in gram amounts. The agonist of the instant invention may be advantageously produced at generally reduced cost, as compared with conventional techniques.

Further aspects of the present invention include host cells and germ cells transformed according to the present invention as well as the essentially pure hybrid chimeric proteins produced according to the present invention.

Brief Description of the Figures

Further undertaking may be had by study of the accompanying figures wherein:

Figure 1 shows the nucleotide and amino acid sequence of equine α cDNA;

Figure 2 shows the nucleotide and amino acid sequence of bovine β FSH;

Figure 3 shows bovine Papilloma virus based expression vector with BamH1 and BglII cloning site.

Detailed Description of Invention and Best Mode

In order to produce the essentially pure hybrid hormones in accordance with the present invention, a eukaryotic host cell is transformed with a vector comprising a promoter region, a first DNA sequence coding for an equine α subunit of a hormone which has the common α subunit of the gonadotropins, and a second DNA sequence coding for a β subunit of a hormone which has the common subunit of the gonadotropins, from a non-equine animal species. The first and second DNA sequences are in transcriptional reading frame with the promoter. The transformed eukaryotic host cells are then cultured under conditions which allow production of the hybrid hormone. The eukaryotic cell is preferably a mammalian cell and the vector preferably further includes a DNA sequence providing for self-replication.

In the most preferred embodiment, mammalian cell expressions that employ mouse C127 cells (epithelioid), capable of co- and post-translational modification of the molecules encoded by the cDNAs inserted within the expression vector, will be used. Equivalent amounts of a vector containing the equine alpha cDNA and a vector containing the bovine FSH (or LH) cDNA will be ideally transfected into C127 or other suitable host cells. Transformed cells will be identified by their characteristic piled-up appearance. The cell lines are then ideally expanded, culture producing within the culture medium, and large amounts of the recombinant DNA derived hybrid equine/bovine FSH which can then be purified.

Example 1: Equine Alpha Subunit

The cDNA clone for the equine common alpha subunit was isolated from an equine cDNA library constructed in the plasmid pBR322 at its PstI site by G-C tailing. Fresh horse pituitary glands were obtained within 10 minutes of slaughter by dissection and quick-frozen in liquid nitrogen. RNA was prepared from the tissue by pulverization and homogenization in a 1:1 mixture of phenol:100mM Na-acetate (pH=5.2) that contained 0.5% SDS at 65°C. After quick cooling on ice, the phases were separated by centrifugation at 5000 X 9 for ten minutes. The hot phenol extraction was repeated twice more followed by two extractions with chloroform : isoamyl alcohol (24:1). RNA was precipitated from the final pooled aqueous phase by the addition of 2.5 volumes of ethanol at -20°C.

Poly A+ messenger RNA was separated from total pituitary RNA by chromatography across an oligo (dT) - cellulose column. The mixture was passed over the column in 10mM Tris-HCl (pH=7.5) containing 0.5M NaCl and washed several times with this buffer. Poly A+ mRNA was eluted with 10mM Tris-HCl (pH=7.5) containing 1mM EDTA and 0.05% SDS.

The cDNA library was constructed from the poly A+ mRNA by standard procedures using i) avian myeloblastosis virus (AMV) reverse transcriptase to prepare first strand cDNA, and ii) both AMV reverse transcriptase and the Klenow fragment of the E.coli DNA polymerase to prepare the second strand. Following treatment with S1 nuclease, the double stranded cDNA was tailed by the addition of dC residues to the 3' hydroxyl group by the use of the

calf-thymus deoxynucleotidyl transferase. Tailed cDNA was annealed to dG-tailed pBR322 and the mixture was used to transform E.coli MC1061 (available from the American Type Culture Collection, Rockville Maryland 20852) to tetracycline resistance.

The library was screened with a ^{32}P labeled bovine alpha cDNA probe purchased from R. Maurer at the University of Iowa. Positively hybridizing clones were isolated and grown up for characterization by restriction enzyme digest and nucleotide sequencing. The nucleotide sequence and predicted alpha subunit protein are provided in Figure 1.

Example 2: Bovine FSH Beta

The bovine FSH beta subunit cDNA was obtained from a bovine cDNA library that was established by methods substantially the same as those described for equine in Example 1. The library was screened with a partial FSH beta clone purchased from Dr. R. Maurer (from the University of Iowa). Full length clones were identified, sequenced and amino acid predictions were made as shown in Figure 2.

Example 3: Expression Vector

Each of the isolated and purified cDNAs (for equine alpha and bovine FSH beta) was inserted into the expression cassette as detailed in Figure 3. This mammalian cell expression vector advantageously contains the bovine papilloma virus (BPV) genome. the rat metallothionein gene promoter region, a unique

cloning site for insertion of the cDNA of interest and metallothionein-poly A sequences. The construction of this vector is not critical; those skilled will recognize that a plethora of alternatives abound which can be used in substitution. It will be recognized that conventional techniques such as those described in Maniatas, "Molecular Cloning, A Laboratory Manual", will readily suffice.

Example 4: Transfection of Cells

To produce cell lines that synthesize and secrete the hybrid FSH molecule, C127 mouse epithelioid cells are transfected with a mixture of two plasmids, the equine alpha cDNA in the metallothionein-BPV plasmid and the bovine FSH cDNA in this same vector. This is ideally accomplished as follows. Ten micrograms of each plasmid are added to 0.5ml of a 250mM $CaCl_2$ solution containing 10µg of salmon sperm DNA as a carrier. The mixture is bubbled into 0.5ml of 280 mM NaCl, 50mM HEPES and 1.5 mM Na phosphate. The calcium phosphate precipitate is allowed to form for 30 minutes at 23°C.

Twenty-four hours prior to transfection, $5X10^5$ mouse C127 cells are transferred to a 100mm culture dish. Immediately before adding exogenous DNA, the cells are fed with fresh culture medium containing 10% fetal bovine serum. One ml of the calcium phosphate precipitate is added to each dish (10ml) and the cells are incubated at 37°C for 8h. The medium is aspirated and replaced with 5ml of 20% glycerol in phosphate-buffered saline (PBS) (pH=7.0) for two minutes at room temperature. The cells are

washed once with PBS, fed with 10ml of medium and incubated at 37°C for 24h. Within 3 weeks, foci of transformed cells, characterized by their piled-up appearance, are identified, transferred to T-25 flasks and line expansion begun.

The equine alpha/bovine FSH dimer is ideally purified by a three step process including trisacryl Blue chromatography, ion exchange and gel filtration. Following purification and analysis of the product by a bovine beta FSH-specific radioimmunoassay, a series of in vitro FSH bioassays can optionally be performed to determine the relative potency of this hybrid molecule compared with recombinant equine, bovine and human FSH. In addition, in vivo bioassays can optimally be performed to determine the potency of each of the preparations in a rodent model. The hybrid molecule will ideally exhibit enhanced biopotency.

While the preferred examples of the present invention use equine alpha subunit and bovine FSH beta subunit, those of ordinary skill in the art will understand that the present invention can be practiced in a similar manner using the beta subunit from any protein which has the common alpha subunit of the gonadotropins. This includes not only the gonadotropins themselves, but also thyroid stimulating hormone (TSH) which also shares the common alpha subunit of the gonadotropins. Furthermore, the beta subunit may be from any desired species. In all cases the presence of the equine alpha subunit will cause a great increase in the bio-activity of the hormone of the beta subunit in the species from which that beta subunit was taken. Thus, for example, if it

is desired to produce a human FSH with greatly enhanced bioactivity, a recombinant chimeric molecule can be produced in the manner discussed above to provide analpha equine subunit bound to the human FSH beta subunit. Accordingly, in its broadest sense, the present invention relates to the production of recombinant hybrid chimeric gonadotropin hormones (or hormones having the common alpha subunit of the gonadotropin hormones) having an equine alpha subunit and the beta subunit of the hormone whose activity it is desired to increase.

It should further be understood that the hybrid molecules of the present invention produced by recombinant DNA technology can be produced in essentially pure form and, in particular, they are produced without contamination by other hormones and hormone subunits. Thus, the essentially pure hybrid molecules of the present invention are novel as prior art hybrid gonadotropins produced with classical protein chemistry techniques cannot be obtained in essentially pure form and, in particular, cannot be obtained without contamination with other hormones and hormone subunits. Thus, the essentially pure hybrid hormones of the present invention are novel and obtaining such novel hormones would not have been obvious to one of ordinary skill in the art having knowledge only of the classical protein chemistry techniques for the production of such hormones.

Those skilled in the art will readily recognize that numerous departures may be made from the procedures taught in the example without departing from either the spirit or scope of the present invention.

What is Claimed is:

1. A method for producing a hormone super-agonist, comprising:

a) transforming eukaryotic host cells with a vector comprising a promoter region and a first DNA sequence coding for an equine α subunit of a hormone which has the common α subunit of the gonadotropins and a second DNA sequence coding for a β subunit of a hormone which has the common subunit of the gonadotropins, from a non-equine animal species, said first and second DNA sequences being in transcriptional reading frame with said promoter; and

b) culturing said transformed eukaryotic host cells under conditions which allow production of the hormone super-agonist.

2. The method of Claim 1 wherein said vector further comprises a DNA sequence providing for self-replication.

3. The method of Claim 1 wherein said hormone having the common α subunit of the gonadotropins is chorionic gonadotropin, follicle stimulating hormone, luteinizing hormone or thyroid stimulating hormone.

4. The method of Claim 1 wherein said non-equine animal species is bovine.

5. The method of Claim 3 wherein said second DNA sequence codes for the β subunit of a bovine gonadotropin selected from the group consisting of FSH and LH.

6. The method of Claim 5 wherein said vector furthering comprises at least a portion of the bovine papilloma virus genome.

7. The method of Claim 6 wherein said promoter region comprises a metallothionein gene promoter region.

8. The host cell transformed according to the method of Claim 1.

9. The host cell of Claim 8 being a mammalian host cell.

10. The host cell of Claim 9 being a C127 cell.

11. The hormone super-agonist produced by the host cell of any of claims 8 to 10.

12. A hormone super-agonist in essentially pure form, free of contamination from other hormones or hormone subunits, comprising a hybrid chimeric molecule with an equine α subunit of a hormone having the common α subunit of the gonadotropins and a non-equine β subunit of a hormone having the common α subunit of the gonadotropins.

13. A hormone super-agonist in accordance with claim 12, wherein the hormone of said equine α subunit is not the same as the hormone of said non-equine β subunit.

14. A hormone super-agonist in accordance with claim 12, wherein said non-equine β subunit is from a bovine, ovine, porcine or human gonadotropin or thyroid stimulating hormone.

FIGURE 1

EQUINE ALPHA SUBUNIT cDNA AND AMINO ACID SEQUENCE

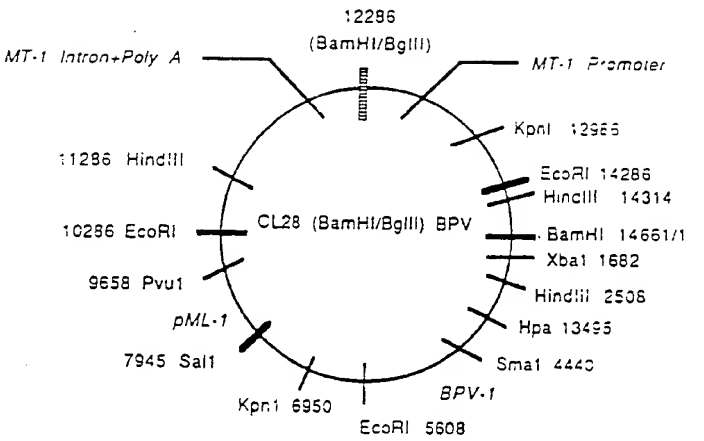
```
   1  GGGGGGGGGGGGGGGGGGGCTGCTCTGAACACATCCTACAAAAAGTCCAGAGGAAGAAG   60

      MetAspTyrTyrArgLysHisAlaAlaValIleLeuAlaThrLeuSerValPheLe
  61  AGCCATGGATTACTACAGAAAACATGCAGCTGTCATCCTGGCCACATTGTCCGTGTTTCT  120

      uHisIleLeuHisSerPheProAspGlyGluPheThrThrGlnAspCysProGluCysLy
 121  GCATATTCTCCATTCCTTTCCTGATGGAGAGTTTACAACGCAGGATTGCCCAGAATGCAA  180

      sLeuArgGluAsnLysTyrPhePheLysLeuGlyValProIleTyrGlnCysLysGlyCy
 181  GCTAAGGGAAAACAAGTACTTCTTCAAACTGGGCGTCCCGATTTACCAGTGTAAGGGCTG  240

      sCysPheSerArgAlaTyrProThrProAlaArgSerArgLysThrMetLeuValProLy
 241  CTGCTTCTCCAGAGCGTACCCCACTCCAGCAAGGTCCAGGAAGACAATGTTGGTCCCAAA  300

      sAsnIleThrSerGluSerThrCysCysValAlaLysAlaPheIleArgValThrValMe
 301  GAACATCACCTCAGAATCCACATGCTGTGTGGCCAAAGCATTTATCAGGGTCACAGTCAT  360

      tGlyAsnIleLysLeuGluAsnHisThrGlnCysTyrCysSerThrCysTyrHisHisLy
 361  GGGAAACATCAAGTTGGGAGAACCACACCCAGTGCTATTGCAGCACTTGCTATCACCACAA 420

      sIleEnd
 421  GATTTAAATGTTTCACCAAGTGCCTTGTGGATGACTGCTGATTTCCACCCCCCCCCCCCCC 480
```

FIGURE 2

BOVINE FSH BETA cDNA NUCLEOTIDE SEQUENCE AND AMINO ACID SEQUENCE

```
                                                  MetLysSerValGlnPheCysPheLeuPhe
1   GTCAGCATCTACAGTTATCAAGTGCCCAGGATGAAGTCTGTCCAGTTCTGTTTCCTTTTC   60

    CysCytTrpArgAlaIleCysCysArgSerCysGluLeuThrAsnIleThrIleThrVal
61  TGTTGCTGGAGAGCAATCTGCTGCAGAAGCTGCGAGCTGACCAACATCACCATCACGGTG  120

    GluLysGluGlyCysGlyPheCysIleSerIleAsnThrThrTrpCysAlaGlyTyrCys
121 GAGAAAGAGGAATGTGGCTTCTGCATAAGCATCAACACCACGTGGTGTGCAGGCTACTGC  180

    TyrThrArgAspLeuValTyrArgAspProAlaArgProAsnIleGlnLysThrCysThr
181 TACACCCGGGACTTGGTGTACAGGGACCCAGCAAGGCCCAATATCCAGAAAACGTGTACC  240

    PheLysGluLeuValTyrGluThrValLysValProGlyCysAlahisHisAlaAspSer
241 TTCAAGGAGCTGGTCTACGAGACGGTGAAAGTGCCTGGCTGTGCTCACCATGCAGACTCC  300

    LeuTyrThrTyrProValAlaThrGluCyshisCysSerLysCysAspSerAspSerThr
301 CTGTACCGTACCCAGTAGCCACTGAATGTCACTGACAGCAAGTGCGACAGCGACAGCACT  360

    AspCysThrValArgclyLeuGlyProSerTyrCysSerPheArgGluIleLysGluEnd
361 GACTGCACCGTGCGAGGCCTGGGGCCCAGCTACTGCTCCTTCAGGGAAATCAAAGAATAA  420

421 AGAGCACGGGATGCTTTGAGCTGCCTACCCTTATCCTAAAGGACCAAAACATCCAAGATG  480

481 TCTGTGTGTACATGTGCGTAGGCTGCAGACCACCACGGGAGACCCTACTGACCTCTGCTC  540

541 TCCTGAC  547
```

Figure 3


12286
(BamHI/BglII)
MT-1 Intron+Poly A
MT-1 Promoter
KpnI 12986
11286 HindIII
EcoRI 14286
HincIII 14314
CL28 (BamHI/BglII) BPV
10286 EcoRI
BamHI 14661/1
Xba1 1682
9658 Pvu1
HindIII 2508
pML-1
Hpa 13495
7945 Sal1
Sma1 4440
BPV-1
Kpn1 6950
EcoRI 5608

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 89/01017

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 P 21/02, C 12 N 15/85, C 12 N 15/16, C 07 K 15/06

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Molecular and Cellular Endocrinology, vol. 40, 1985, Elsevier Scientific Publishers Ireland, Ltd (Shannon, IR), G.R. Bousfield et al.: "Hybrids from equine LH: alpha enhances, beta diminishes activity", pages 69-77, see the whole article	1-11
X	cited in the application	12-14
Y	Journal of Endocronology, vol. 115, 1987, Journal of Endocronology Ltd (London, GB), F. Stewart et al.: "Nucleotide (cDNA) sequence encoding the horse gonadotrophin alpha-subunit", pages 341-346, see figure 1; page 345, column 1, lines 53-58	1-11
Y	WO, A, 85/01958 (REDDY et al.) 9 May 1985, see claims	1-11

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th November 1989	05 DEC 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 8901017 SA 30605

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/11/89 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8501958	09-05-85	US-A- 4840896	20-06-89
		EP-A- 0160091	06-11-85
		EP-A- 0160699	13-11-85
		JP-T- 61500249	20-02-86

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February 14, 1997

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Re: PCT/US96/06483 International Patent Application of The Government of the United States of America "GLYCOPROTEIN HORMONE SUPERAGONISTS" (Szkudlinski *et al.*)
Our Ref.: 14014.0185/P
DHHS Ref.: E-015-96/0

Dear J.P.:

Enclosed is a copy of the PCT International Search Report dated January 29, 1997, and the references cited therein. We are pleased to note that no reference particularly relevant to the present invention was found by the search, and therefore, amending the claims in response to this Search Report is not necessary. Please note that we will have another opportunity to amend the entire application, if necessary, after the issuance of a Written Opinion.

If you have any questions, please do not hesitate to contact me.

Very truly yours,

NEEDLE & ROSENBERG, P.C.

David G. Perryman

DGP/MAW:lb
Enclosures
cc: Mariusz W. Szkudlinski, M.D., Ph.D. (w/enc.)
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